Decreased hippocampal cell proliferation in mice with experimental antiphospholipid syndrome

Frauenknecht, Katrin ; Leukel, Petra ; Weiss, Ronen ; von Pein, Harald D ; Katzav, Aviva ; Chapman, Joab ; Sommer, Clemens J

Abstract: The antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of antiphospholipid antibodies, which may trigger vascular thrombosis with consecutive infarcts. However, cognitive dysfunctions representing one of the most commonest neuropsychiatric symptoms are frequently present despite the absence of any ischemic brain lesions. Data on the structural and functional basis of the neuropsychiatric symptoms are sparse. To examine the effect of APS on hippocampal neurogenesis and on white matter, we induced experimental APS (eAPS) in adult female Balb/C mice by immunization with 2-glycoprotein 1. To investigate cell proliferation in the dentate gyrus granular cell layer (DG GCL), eAPS and control mice (n = 5, each) were injected with 5-bromo-2 -deoxyuridine (BrdU) once a day for 10 subsequent days. Sixteen weeks after immunization, eAPS resulted in a significant reduction of BrdU-positive cells in the DG GCL compared to control animals. However, double staining with doublecortin and NeuN revealed a largely preserved neurogenesis. Ultrastructural analysis of corpus callosum (CC) axons in eAPS (n = 6) and control mice (n = 7) revealed no significant changes in CC axon diameter or g-ratio. In conclusion, decreased cellular proliferation in the hippocampus of eAPS mice indicates a limited regenerative potential and may represent one neuropathological substrate of cognitive changes in APS while evidence for alterations of white matter integrity is lacking. Keywords Antiphospholipid syndrome Corpus callosum g-ratio BrdU Neurogenesis

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Brain Structure and Function

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	and control mice (n=7) revealed no significant changes in CC axon diameter or g-ratio. In conclusion, decreased cellular proliferation in the hippocampus of eAPS mice indicates a limited regenerative potential and may represent one neuropathological substrate of cognitive changes in APS while evidence for alterations of white matter integrity is lacking.

Dear Professor Zilles,

Firstly, we would like to thank for the extension of the resubmission deadline. Furthermore, we are very grateful for the critical comments of the reviewers. In fact, there was a mistake in the statistics, as fortunately detected by reviewer #1. For analysis of the axon values, the individual measurements from the same animals were falsely considered as independent observations. This was corrected and resulted in no significant differences in the various axonal parameters. We critically re-evaluated the manuscript and re-wrote the respective passages in the Discussion. Despite these changes we feel the results are important and improve our understanding of the cognitive decline in APS in the absence of any ischemic lesion.

All changes in the revised mansucript have been marked in yellow. We hope that the modified manuscript will now be acceptable for publication in ***Brain Structure and Function***.

Sincerely Yours

Katrin Frauenknecht

Reviewer #1: Frauenknecht et al have investigated the effects of aPL on the hippocampus. Unfortunately it is difficult to follow exactly why this study was performed, more specifically what specific aspects of the human disease are reproduced using this animal model, and how these results improve our understanding of the human disease, since this was the goal. Some important information is missing from the materials section, which had led me to make some assumptions about the results. If my assumptions are wrong I apologise, and I hope the authors can clarify the text further.

Introduction

The introduction cites literature on a variety of animal models for inflammatory diseases, explaining how the authors came to the decision to study animals injected with b2 glycoprotein. What I do not understand is what exact feature of the human APS they are aiming to model and understand in their studies.

We thank the reviewer for the critical comment. In our revised version we partly re-wrote the Introduction for more clarity. It is now explicitly stated why we chose the model used and that we focused on the structural and molecular basis for the cognitive decline which is seen both in many human APS patients and in mice with eAPS.

The authors indicate that at present it is unclear whether aPL directly or indirectly affects CNS integrity. This question remains is not targeted by the study, since the observed effects are equally likely to be direct as indirect. This is confusing.

We agree with the reviewer and removed this text passage.

The authors say in the introductions that SLE animal models display psychiatric manifestations. This should be rephrased. I suspect that the authors intended to say that the animals display behavioural abnormalities reminiscent of psychiatric problems. Animals don't have psychiatric problems.

The reviewer is right. We replaced “psychiatric” by “behavioral”.

M&M

What age or weight were the animals?

The mice used in the present study were immunized at the age of eight weeks and were then investigated in the staircase test at the age of 24 weeks for behavioral alterations (sixteen weeks post immunization (p.i.)). We have mentioned the age at immunization in the material and methods section.

Please change endogen peroxidase into endogenous peroxidase. Please change 2N into the more modern 2M HCl.

The wording has been modified accordingly.

A correction is performed for missing sections using a correction factor. However, this correction factor is based on the number of sections missing, but it does not take into account that the levels of the sections may influence the results. Why not use Cavalieri's principle to estimate the number of cells in the DG?

We completely agree with the reviewer that an optical fractionator method would be optimal. The problem are some missing sections due to artefacts, which makes this kind of analysis impossible (e.g. Shors et al, Nature, 2001;410:372-376). The correction factor used allows at least a quite good approach for the estimation of the number of DG cells.

How are the calculations presented in Figure 4 performed?

As suspected by the reviewer (c.f. query to “Figure “), the analysis of the axon values was based on individual measurements from the same animals and were falsely considered as independent observations. In fact, we are very grateful for the critical reading of this reviewer. After correction of this mistake significance level was lost. Nevertheless, we feel this is an important finding and modified the manuscript accordingly.

Results

The authors state that the number of DCX-BrdU double positive cells was reduces, as well as the NeuN-BrdU double positive cells. This is not the case according to their own set level of significance. There is no difference, and the data should therefore not be interpreted as such.

As requested, we modified the manuscript accordingly.

Figure 2

The limited information presented in figure 2 does not require a whole figure. Therefore I would suggest removing this figure all together.

As suggested, figure 2 has been removed.

Figure 4

As said it is not clear how these results were calculated. Are the individual measurements considered independent observations, even though they were from the same animals? This would not be correct, but would explain the small error bars.

As mentioned above (c.f. answer to "How are the calculations presented in Figure 4 performed?"), the analysis of the axon values was based on individual measurements from the same animals and were falsely considered as independent observations. After correction of this mistake significance level was lost. Nevertheless, we feel this is an important finding and modified the manuscript accordingly.

Discussion

First sentence, again neuropsychiatric abnormalities would not be my description of choice for aberrant animal behaviour.

As suggested, the wording has been changed.

I expected it to become clear here what exactly the behavioural changes are that occur in human APS. What are these neurological manifestations exactly?

There is a wide spectrum of behavioral changes in human APS but cognitive dysfunctions are most frequently reported. We stated this now more precisely in the Introduction.

Another important finding that partly explains the ….. (fifth paragraph of the discussion). Cognitive deficits may be related to reduced myelin, but I do not agree that the reduced myelin explains the cognitive deficits. The same holds for the final sentence of the conclusion. There is no direct evidence provided that the changes in white matter are the cause of cognitive impairment. This is an overinterpretation of the findings.

As mentioned above we did no longer see significant differences in the white matter of eAPS mice after eradication of the mistake in the statistics. The Discussion has been re-written.

Reviewer #3: This is a very well written paper on an extremely interesting topic. Using an animal model the authors examined the influence of APS (antiphospholipid syndrome) on hippocampal neurogenesis and on white matter volume. For this they induced experimental APS (eAPS) in adult female Balb/C mice by immunization with β2-glycoprotein 1. Sixteen weeks after immunization, eAPS resulted in a significant reduction of BrdU positive cells in the DG GCL compared to control animals. However, double staining with doublecortin (DCX) and NeuN revealed a largely preserved neurogenesis. In summaryy, this is a well-done, well-written, and important paper reporting interesting results. Thus, this paper definitively deserves publication. I have listed my detailed comments and suggestions below.

Introduction

The intro is fine. The study question has also been formulated appropriately.

Methods

Methods are state-of-the-art.

Statistical tests: Why did they only use t-test and not a multivariate variant of the t-test (e.g., Hotteling's t test)? Did they control for multiple comparisons (obviously not)?

As suspected by the reviewer we did not correct for multiple testing which would be required e.g. for the Hotelling test. We are aware of a potential alpha error culmination in this case. On the other hand, multivariate testing also goes along with some problems in particular to comply with variances with respect to the multidimensional prerequisites given.

I would strongly recommend to report also effect size measures. In this case Cohen's d measure would be most appropriate. Effect size measures are important to evaluate an effect independent from sample size especially when sample size are small (as in this case). Maybe the authors consider to apply Baysian statistics, which is a bit more appropriate in this case.

As suggested, we calculated effect sizes of our data according to Cohen. The values are included in the Results.

Results

Result presentation is fine. However, what does the ± mean (SD, SE, or CI)?

The ± means SEM. The information is given in the paragraph statistical analysis in Materials and Methods. The abbreviation SEM has been included in the corresponding list.

Reviewer #4: Frauenknecht et al. provide an interesting analysis of hippocampal and ultraxtrucutral changes of myelinated callosal axons in an animal model of the antiphospholipid syndrome (APS).

The authors conclude that the neuropathological changes in the experimental APS model might be due to a decreased cellular proliferation in the hippocampus and alterations of the white matter integrity.

Due to the still existing poor understanding of neuropsychological symptoms in APS patients, this study is of interest to the readership of BSAF. It is well conducted, the results are well described and the discussion mentions potential problems and limitations. The conclusions of the authors are supported by the data described.

Though this is a well written and comprehensive manuscript of interest, I would like to address a few minor points:

Minor points

1. The authors should describe the ELISA used for the detection of antiphospholipid antibodies (aPLs) more detailed and indicate whether cardiolipin is the only autoimmune target in the METHODS section and FIGURE 1. After all, immunization of mice was done with beta2 glycoprotein I.

We completely agree with the reviewer that the immunization of mice was done with β2-GPI alone. The ELISA technique (according to Bakimer et al. 1992) used in our study measures β2-GPI-dependent anti-Cardiolipin antibodies (aCL) and no other targets. We have mentioned that in the Material & Methods sections and in Figure 1, respectively.

2. INTRODUCTION section: The authors should mention that aPL are a persistent marker of APS patients and discuss the possibility of intrathecal production of aPL. aPL may interact with pure phospholipids or co-factors thereof like beta2 glycoprotein I. Both targets can induce different signaling pathways.

As suggested, we briefly mentioned this issue at the end of the first paragraph of the Introduction.

3. The abbreviation OD should be given in the corresponding list.

The abbreviation OD has been included in the list.

Decreased hippocampal cell proliferation in mice with experimental antiphospholipid syndrome

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Authors' contributions: Experimental design: KF, AK, CJS; Animal experiments: AK, RW; Histology/Volumetry/Morphometric studies: KF, PL, HDP; Figures: KF, PL, HDP; Manuscript KF, PL, JC, CJS. All authors read and approved the final manuscript. The authors declare no competing interests.

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Ethical approval: Animal experiments have been approved Israeli Health Ministry (Ethical approval no.775/12) and by the Chaim Sheba Medical Center Animal Welfare Committee. All experiments were in accordance with national and international guidelines and regulations. This article does not contain any studies with human participants performed by any of the authors.

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Abstract

The Antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of antiphospholipid antibodies (aPL), which may trigger vascular thrombosis with consecutive infarcts. However, cognitive dysfunctions representing one of the most commonest neuropsychiatric symptoms are frequently present despite the absence of any ischemic brain lesions. Data on the structural and functional basis of the neuropsychiatric symptoms is sparse. To examine the effect of APS on hippocampal neurogenesis and on white matter, we induced experimental APS (eAPS) in adult female Balb/C mice by immunization with β2-glycoprotein 1. To investigate cell proliferation in the dentate gyrus granular cell layer (DG GCL), eAPS and control mice (n=5, each) were injected with 5-bromo-2'-deoxyuridine (BrdU) once a day for 10 subsequent days. Sixteen weeks after immunization, eAPS resulted in a significant reduction of BrdU positive cells in the DG GCL compared to control animals. However, double staining with doublecortin (DCX) and NeuN revealed a largely preserved neurogenesis. Ultrastructural analysis of corpus callosum (CC) axons in eAPS (n=6) and control mice (n=7) revealed no significant changes in CC axon diameter or g-ratio. In conclusion, decreased cellular proliferation in the hippocampus of eAPS mice indicates a limited regenerative potential and may represent one neuropathological substrate of cognitive changes in APS while evidence for alterations of white matter integrity is lacking.

Keywords: Antiphospholipid syndrome, corpus callosum, g-ratio, BrdU, neurogenesis, white matter.

Introduction

In the antiphospholipid syndrome (APS), stroke is one of the most common causes of disability and death (Cervera et al. 2009; Cervera et al. 2015). However, apart from thrombotic events in APS various non-stroke neuro-psychiatric manifestations are consistently observed (Yelnik et al. 2016). In this very heterogeneous group of syndromes cognitive dysfunctions are most frequently reported (Conti et al. 2012; Jacobson et al. 1999; Tektonidou et al. 2006; Yelnik et al. 2016). Although certain aPL types and levels seem to be strongly associated with non-thrombotic neurological symptoms (Stojanovich et al. 2013; Jacobson et al. 1999), the underlying mechanisms remain to be clarified. While aPL are persistent markers in APS patients it is currently under discussion whether these autoantibodies may also be produced intrathecally suggesting a central nervous system independent autoimmune process (Sokol et al. 2007).

When focusing on the structural and molecular basis of the cognitive alterations in APS the experimental model of APS (eAPS) with β2-Glycoprotein 1 (β2-GPI) immunized mice represents a valuable tool (Shrot et al, 2002). After immunization, these mice show increased aPL titers and develop a hyperactive and cognitively impaired phenotype in the absence of any ischemic features in routine histology (Frauenknecht et al. 2015; Shrot et al. 2002). Nevertheless, additional investigations have revealed a regional breakdown of blood-brain barrier (BBB) integrity and IgG accumulation in neurons (Katzav et al. 2014) as well as alterations in 5-HT1A receptor binding densities, especially in the hippocampus (Frauenknecht et al. 2013). Furthermore, there is evidence of decreased synaptic spine density in hippocampal CA1 pyramidal neurons and a reduction of synaptopodin protein expression in the hippocampus (Frauenknecht et al. 2015).

Although inflammation and a distinct CNS phenotype are absent in eAPS at this stage of disease (Frauenknecht et al. 2013 and 2014), dendritic atrophy and dendritic spine loss are features reminiscent of mouse models of systemic lupus erythematosus (SLE) (Sakic et al. 1998; Sakic et al. 2000). Frequently, animal models of SLE display neurologic and behavioral manifestations, but the pathological changes observed in the brain are much more impressive than the changes seen in the eAPS model (Denenberg et al. 1992; Ma et al. 2006; Sakic et al. 1998; Sakic et al. 2000). One might speculate that the changes seen in eAPS mice may reflect a form of subtle autoimmunity-induced neurodegenerative disease.

To further elucidate the structural and molecular changes underlying the cognitive decline in APS in the absence of stroke we used the well characterized mouse model of APS (eAPS) by immunization with β2-GPI and lack of ischemic damage (Shrot et al. 2002). In our present study we first addressed the question of whether reduced dendritic complexity in the hippocampus may be associated with changes of the hippocampal volume. We further hypothesized that long exposure to aPL in eAPS may have an impact on the proliferation of cells as well as on neuroblast differentiation as it has been shown for other autoimmune disease models such as lupus (Kapadia et al. 2012; Stanojcic et al. 2009) and experimental autoimmune encephalomyelitis (EAE) (Huehnchen et al. 2011). For this purpose, we investigated the number of proliferating cells and generation of newborn neurons in the subgranular zone / granular cell layer of dentate gyrus of the hippocampus in eAPS and control mice. White matter abnormalities have been suggested to play a role in the pathophysiology of cognitive deficits (Filley 2010; Penke et al. 2012). We therefore expanded our analyses to include ultrastructural examination of callosal myelinated fibers in eAPS mice.

Material and methods

Animal experiments and tissue processing for hippocampus volumetry and immunohistochemistry/immunofluorescence

Animal experiments have been approved Israeli Health Ministry (Ethical approval no.775/12) and by the Chaim Sheba Medical Center Animal Welfare Committee. All experiments were in accordance with national and international guidelines and regulations. Female Balb/C mice (n=10) were obtained from Harlan Laboratories Limited (Israel) and housed under standard conditions (23°C ± 1°C, 12 h light cycle (7 am ± 7 pm) with ad libitum access to food and water (Animal facility, Chaim Sheba Medical Center, Tel Hashomer).

For analysis of hippocampus volume, cell proliferation and neurogenesis in the DG GCL female BALB/c mice at the age of 8 weeks were immunized once with 10 ug β2 GPI emulsified in complete Freund's adjuvant (CFA) (n=5) while controls were immunized with CFA alone (n=5). For Measurement of aPL levels (β2-GPI-dependent aCL), 96-well plates were coated with cardiolipin (50 ug/ml in ethanol) and the ELISA technique was used as previously described (Bakimer et al. 1992).

Hyperactive behavior within a 3 min period was assessed 16 weeks after immunization by the staircase test as described (Pick et al. 1996; Simiand et al. 1984). After determination of aCL levels and hyperactive behavior in the staircase test, mice were injected with BrdU (100 mg/kg KG; Sigma-Aldrich Cat. B5002) once a day for ten subsequent days to assess cellular proliferation over a longer period. After the last BrdU injection, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) followed by transcardially perfusion with PBS followed by 4% paraformaldehyde (PFA) in PBS. The brains were carefully removed,

post-fixed in 4% PFA at 4 °C overnight and stored in 0.5% PFA. Brains were sliced into 40-µm coronal sections using a vibratome (VT1000S, Leica, Germany). Sections were collected in series of 6 throughout the entire rostral-caudal extent of the hippocampus. From each series the first section was Nissl-stained according to standard protocols and was then used for volumetry as described below. Section 2 was subjected to DCX-Immunohistochemistry, section 3 to BrdU-Immuno-fluorescence, section 4 to BrdU and DCX double staining and section 5 was used for BrdU and NeuN double staining.

For determination of hippocampus volume, Nissl-stained sections were scanned at a magnification of x1.6 with a Leica Microscope (Leica, Wetzlar, Germany) and digitized. Using the MCID image analysis system (Imaging Research Inc, St. Catharines, Ontario, Canada), hippocampal volumes were calculated by multiplying sectional areas by interval thickness and expressed as hippocampus volume in mm^3 ± SEM.

For DCX immunohistochemistry (IHC) free-floating 40-µm-thick vibratome sections were incubated in 1 ml H_2O_2 (30%) in 100 ml Methanol / TBS (1+1) to block endogenous peroxidases. Thereafter, the sections were washed for 10 min with TBS and twice for 10 min with TBS-T (0.2 % Triton-X-100) followed by a 30 min incubation step (5 % normal goat serum (NGS)/TBS). Free floating sections were incubated with anti-DCX antibody (Rabbit polyclonal anti-DCX antibody; ab18723; 1:2000 in 5% NGS/TBS; Abcam, UK) followed by 30 min incubation (RT) with biotinylated anti-rabbit antibody (1:50 5% NGS/TBS; Linares, Germany). After rinsing with TBS-T (4x 10 min), immunoreactivity was visualized by the avidin–biotin–peroxidase complex method (Vectastain Kit Universal, Vector laboratories, UK) followed by 10 min washing-steps with TBST and TBS. Subsequently, sections were developed with the DAB Labvision Kit (Thermoscientific, Germany) for 30 sec at room temperature, rinsed with TBS, wet-

mounted onto slides, dehydrated and cover-slipped using Cytoseal XYL (Thermoscientific, Germany). Negative controls were obtained by omitting the primary antibody.

BRDU/DCX double-staining immunofluorescence was performed as follows: 40 µm-thick vibratome sections were washed for three times in TBS, incubated in 50% formamid/2xSSC for 1.5 h at 65°C followed by two washing steps with 2xSSC. Afterwards, free floating sections were incubated in 2M HCl for 30 min at 37°C and then neutralized with 0.1 M borat buffer pH 8.5. Sections were washed in TBS, blocked with 5% NGS in TBST and incubated overnight at 4°C with the anti-BRDU antibody (Rat anti-BrdU antibody; clone BU1/75 (ICR1); 1:500 in 5% NGS/TBS-T; AbD Serotec USA). Thereafter, sections were washed thoroughly in TBS followed by incubation for 2h at room temperature in Alexa Fluor 546 labeled anti-rat antibody (1:200 in 5% NGS/TBS). After that, sections were first washed in TBS and then three times in PBS, followed by blocking in 10% BSA in TBST and incubation overnight at 4°C with the anti-DCX antibody (1:2000 in 5% NGS/TBS; Abcam, UK). Subsequently, sections were washed thoroughly in PBS-Tween (0.25 % Tween 20) followed by incubation for two hours at room temperature with an Alexa Fluor 488 anti-rabbit antibody (1:200 in 10% BSA/PBS). Thereafter, sections were washed in PBS-Tween then three times in PBS followed by incubation for 5 min at room temperature with DAPI (1:1000 in PBS). After washing in PBS, sections were wet-mounted onto slides and immediately cover-slipped using Pro Long Gold antifade. Negative controls were obtained by omitting the primary antibody.

BRDU/NeuN double-staining immunofluorescence was performed as in BrdU/DCX double staining. After that, sections were first washed in TBS and then three times in PBS followed by blocking in 10% BSA in PBS- 0,2 % Triton X-100 and incubation with

the anti-NeuN antibody (1:500 in 10% BSA, 0,2 % Triton X-100, PBS; clone A60 Millipore, Germany) for 2h at room temperature. Afterwards sections were washed thoroughly in PBS-Tween (0.25 % Tween 20) followed by incubation for two hours at room temperature with an Alexa Fluor 488 anti-mouse antibody (1:400 in 10% BSA/PBS). After washing in PBS, sections were wet-mounted onto slides and immediately cover-slipped using Pro Long Gold antifade. Negative controls were obtained by omitting the primary antibody.

Cell counting

Counting of positively labeled cells was performed by two investigators (KF, PL) blinded to the group assignment of each animal using a Leica DM6000B microscope. Stereological quantification of immunoreactive cells was performed in every 6th section in each DG in the granule cell layer (GCL), including the subgranular zone. Seven sections per staining were used to determine the number of immunoreactive cells. DCX-, NeuN and BrdU-positive as well as double positive cells were counted at x40 using a Leica microscope (Leica, Germany). Due to artificial damage of single DGs, the total number of positively labeled cells **X** per DG GCL per mouse was estimated by multiplying the counted cell number by a correction factor **Z**. **Z** was determined for each animal by dividing the number of 14 DG GCL (2 DG GCL per section multiplied by 7 sections = 14) by the available and undamaged number of DG GCL (**X**) per animal. Cell numbers were then multiplied by **Z** (**Z=14**/**X**). The total number of positive cells per DG GCL animal could be determined by multiplying the corrected cell number by 6.

Tissue processing for light and electron microscopy

For light and electron microscopic assessment of callosal fibers, vibratome sections were obtained from 6 eAPS and 7 control mice brains from our previous study (Frauenknecht et al. 2015). Animal experiments from this previous study have been performed in the same manner as in the present study. One 40µm thick section per mouse at the level of the anterior commissure was fixed in 3% glutaraldehyde for 2 hours at room temperature. Following several rinses in 0.2 M sodium cacodylate buffer (pH 7.3), tissue samples were postfixed in 1% osmium tetroxide in cacodylate buffer for 2 hours. The samples were then dehydrated through an ascending series of ethanol concentrations and embedded in resin with propylene oxide. Semi-thin (0.65 µm) sections for light microscopy and ultrathin (50 nm) sections for electron microscopy were cut in the sagittal plane on a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) starting at midline position (c.f. Fig. 4 a for further details of the tissue processing). Semi-thin sections were stained with methylene blue and viewed with a Leica light microscope (DM2000, Leica Microsystems, Wetzlar, Germany). Ultrathin sections were stained with an alcoholic solution of 1% uranyl acetate and lead citrate in sodium hydroxide and examined with a Zeiss EM-910 transmission electron microscope (Carl Zeiss, Germany). Quantitative electron microscopy was performed on approximately 100 to 200 myelinated fibers/slice randomly photographed at a magnification of 10,000. Total fiber diameter (µm) and axon diameter (µm) were determined using MCID Analysis software. Subsequently, the g-ratio (axon diameter/outer diameter of the myelinated fiber) was calculated. Morphometric analysis of myelinated fibers was performed on 5 images. Further, the number of axons was counted and the axon density determined and expressed as mean number/$\mu m^2 \pm$ SEM.

Statistical Analysis

Statistical analysis was performed using the general statistics module of Analyse-it™ for Microsoft Excel (Analyse-it Software, Ltd., Leeds, UK). Normality was checked using the Shapiro-Wilk test. Significant effects were confirmed by the Student's t-test (normal distribution) or Mann-Whitney test (not normally distributed) with a significance level at $p < 0.05$. Results are presented as mean ± SEM. The investigators carrying out the analyses were blinded to group identity. Additionally, effect sizes were calculated according to Cohen (Cohen 1988) as small (d=0.1), medium (d=0.3) or large (d=0.5).

Results

aPL, Staircase test

Analysis of β2-GPI-dependent aCL antibodies revealed significantly higher ODs as measures of antibody binding in the ELISA assay in eAPS mice compared to controls (Control vs. eAPS: OD 0.0258 ± 0.12951 vs. 1.1162 ± 0.12951; p=0.0003, t-test; d=3.129) (Fig. 1a). In behavioral measures of a 3 min staircase test, eAPS mice showed a significantly higher number of stairs climbed as well as of rearing movements compared to control animals (Control vs. eAPS; stairs: 24.2 ± 1.62 vs. 32.0 ± 1.62; p=0.0093; d=1.034; rearing movements: 22.8 ± 1.75 vs. 28.6 ± 1.75; p=0.0468; t-test; d=0.814) (Fig. 1b).

Hippocampus volumetry

Volume analysis of the hippocampus of eAPS and control mice revealed no significant differences between the experimental groups (Control vs. eAPS: 2.7 mm^3 ± 0.08 vs 2.6 mm^3 ± 0.1; p=0.6142; t-test; d=1.104) (not shown).

Proliferation (BrdU), post-mitotic neuroblasts and immature neurons (DCX), and mature neurons (NeuN)

Overall, the number of BrdU-positive proliferating cells was significantly lower in the DG of eAPS mice (1183 ± 245) compared to control mice (1994 ± 245) (p=0.0473; t-test; d=1.38) (Fig. 2). Interestingly, the number of neuroblasts/immature DCX+ cells was not significantly reduced in eAPS mice DGs compared to control mice (Control vs. eAPS 3791 ± 277 vs. 3677 ± 328; p=0.8200; t-test; d=0.162) (Fig. 2). The number of DCX and BrdU-double positive cells was not significantly different (eAPS mice (492 ± 120) vs. control mice (803 ± 120; p=0.1046; t-test; d=1.046) (Fig. 2). The same was seen for NeuN BrdU-double positive cells (eAPS mice (1022 ± 332) vs. control mice (749 ± 248); p=0.1528; t-test; d=0.53) (Fig. 2).

Electron microscopy

Experimental APS and control mice from our previous study (Frauenknecht et al. 2015) showed largely the same myelinated callosal fiber density/μm^2 (Control vs. eAPS: 1.97 ± 0.13 vs. 1.95 ± 0.14; p=0.9101, t-test). The size frequency distributions for axon and fiber area showed a shift towards larger axon diameter in eAPS mice compared to control mice (Fig. 3) However, overall axon and fiber size as well as g-ratio did not significantly differ (Control vs. eAPS: axon size 0.58 ± 0.019 vs 0.60 ± 0.016 p=0.545, t-test; d=0.349; fiber size 0.80 ± 0.028 vs. 0.81 ± 0.020,p=0.254, t-test; d=0.101;

p=0.179, t-test; d=0.803; 0.71 ± 0.008 vs 0.73 ± 0.002; p=0.179, t-test; d=0.421) (Fig. 3). Myelin abnormalities including demyelination, excessive myelin figures or collapsed myelin sheaths were not detectable.

Discussion

One unresolved question in both human patients and animal models of the antiphospholipid syndrome is the cause of the behavioral abnormalities and cognitive alterations in the absence of an obvious morphological substrate. We could previously show that a reduction of the dendritic complexity of hippocampal CA1 neurons may contribute to cognitive deficits (Frauenknecht et al. 2015). A similar pattern of neuronal atrophy and reduced spine density has been reported in the murine lupus model (Sakic et al. 2000). However, in contrast to the lupus model (Sakic et al. 2000; Sled et al. 2009), long exposure to aPL was not accompanied by any changes in hippocampal volume. This may also explain why associations between aPL levels and hippocampal volume among APS patients with neurological manifestations have not been described.

The major finding of the present study was that cellular proliferation in the hippocampal dentate gyrus of eAPS mice was reduced while neuroblast differentiation remained largely preserved. Continuous adult neurogenesis in the DG is a complex process including multiple steps of cell maturation (Kempermann et al. 2015; Mandyam et al. 2007). Many studies have shown that different pathologic and physiologic stimuli including seizures, stress, learning, and exercise modulate the rate of neurogenesis (Eisch et al. 2008; Gould et al. 1999; Kronenberg et al. 2003; Nakagawa et al. 2000; Parent et al. 2006; van Praag et al. 1999). Reduced numbers of BrdU-positive

proliferating cells in the hippocampus have been shown in rodents after acute and chronic LPS treatment (Bastos et al. 2008; Fujioka and Akema 2010). In autoimmune diseases such as SLE, disturbed cellular proliferation in the DG has been shown in MRL/lpr mice. These mice display decreased BrdU-positive cells in the taste buds indicating impaired cellular renewal (Kim et al. 2012). Using the same animal model, Stanojicic and colleagues (2009) found impaired proliferative capacity in the brain of this lupus-like disease.

To our knowledge no data exists concerning the effect of APS/aPL on hippocampal cell proliferation and neurogenesis. The significant reduction of hippocampal cell proliferation in our present study, reflected by a 59% decrease of BrdU labeled cells in eAPS mice is in accordance with the above-mentioned alterations in the lupus mouse model. As we assessed the numbers of BrdU-labelled cells after 10 days of BrdU treatment, we assume that not only proliferation but also survival of newborn cells is impaired by eAPS. Interestingly, the number of neuroblasts/nascent neurons as well as mature neurons was largely preserved in eAPS mice. This indicates that the differentiation process itself is not affected by aPL exposure. This finding also suggests a compensatory shift towards neuronal maturation despite the decreased regenerative potential. In future studies it would be interesting to find out whether higher levels of aPL or longer exposure to aPL may exceed this capacity, resulting in a decrease of DG neuroblasts and neurons.

The exact mechanism whereby aPL influences cell proliferation is not yet clear. Apart from a direct interaction of aPL with hippocampal progenitor cells leading to a decrease in proliferating cells, there are also hints for indirect mechanisms of action. Altered gene expression and dysregulated Sonic hedgehog, β-catenin and Notch signaling pathways have been shown to modulate hippocampal neurogenesis in experimental

autoimmune encephalomyelitis (Huehnchen et al. 2011). Alternatively, reduced hippocampal cell proliferation in eAPS mice may also be caused by alterations in binding densities of serotonergic 5-HT1A receptors and $GABA_A$ receptors in the hippocampus (Frauenknecht et al. 2013). Both receptors contribute to neurogenesis in the SGZ (Alenina and Klempin 2015; Banasr et al. 2004; Tozuka et al. 2005; Wang et al. 2005). Blockade of 5-HT1A receptors by specific inhibitors or depleption of serotonin has been shown to reduce the number of BrdU+ cells in the SGZ (Klempin et al. 2010; Radley and Jacobs 2002; Ueda et al. 2005).

Apart from the hippocampus, the white matter plays a central role in cognition and transfer of information between different brain regions (Filley 2010; Penke et al. 2012; Urich-Lai et al. 2009). Studies evaluating the effect of aPL / APS on white matter in humans with SLE describe the association of neurological alterations with WM changes that mainly include WM hyperintensities related to ischemic events. (Kaichi et al. 2014; Valdés-Ferrer et al. 2008). However, there is also evidence that WM may be susceptible to elevated aPL titers in the absence of ischemic features. In a study with neurologically asymptomatic female APS patients with obstetric complications without signs of ischemic events, elevated titers for anti-ß2GPI and/or Lupus anticoagulant were associated with subtle white matter changes (Pereira et al. 2016). Using diffusion tensor imaging the authors provided evidence for impairment of myelin and/or axonal structures (Pereira et al. 2016).

In the present eAPS model, neither direct axonal damage nor myelin degeneration was detectable. Although we found ultrastructurally a shift towards larger axon diameter in eAPS, axon diameter and g-ratio did not significantly differ between eAPS and control mice. Thus, there is no evidence that whiter matter changes may be responsible for the cognitive alterations in the eAPS model used.

Conclusion

The present study contributes new information to understanding the biological basis of the behavioral and cognitive anomalies occurring in the absence of ischemic alterations in both APS and the murine disease model.

List of abbreviations

aCL: anti-Cardiolipin antibodies; aPL: antiphospholipid antibodies; APS: Antiphospholipid syndrome; BBB: blood brain barrier; BrdU: 5-bromo-2'-deoxyuridine; BSA: Bovine serum albumin; CA: cornu ammonis; CNS: central nervous system; CC: corpus callosum; CFA: complete Freund's adjuvant; DCX: doublecortin; DG GCL: dentate gyrus granular cell layer; EAE: experimental autoimmune encephalomyelitis; eAPS: experimental antiphospholipid syndrome; LPS: lipopolysaccharide; NGS: normal goat serum; OD: optical density; PBS: phosphate buffered saline; PFA: paraformaldehyde; RT: romm temperature; SEM: standard error of mean; SLE: systemic lupus erythematosus; SSC: saline-sodium citrate; TBS: Tris-buffered saline; TBS-T: TBS with Triton-X-100; WM: white matter;

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Figure Legends

Fig. 1: **Evidence for successful induction of eAPS in mice.**

a) eAPS mice developed significantly higher levels of β2-GPI-dependent anti-Cardiolipin antibodies (aCL) compared to controls immunized with adjuvant alone. **b**) Mice immunized with β2-GPI exhibited the typical behavioral alterations in this ePAS model, namely a significantly higher number of stairs climbed as well as of rearing movements compared to control animals. (* $p<0.05$) abbreviation: OD: optical density

Fig. 2: **Analysis of hippocampal cell proliferation.**

a) BrdU-positive proliferative cell number was significantly lower in the DG of eAPS mice compared to control mice (*$p<0.05$) whereas the number of neuroblasts/immature DCX+ neurons as well as of DCX and BrdU-double positive and of NeuN and BrdU-double positive cells was largely preserved. Representative photomicrographs of BrdU stained sections of Control (**b**) and eAPS (**c**) mice (Scale bar 50 µm) as well as of DCX stained section (**d**) (Scale bar 200 µm; inlet 50 µm) and BrdU and DCX-double positive stained section (**e**) from one control animal (Scale bar 50 µm; inlet 25 µm). Photomicrographs were adjusted to brightness.

Fig. 3: **Ultrastructural analysis of myelinated axons in the corpus callosum**. **a**) gives an impression of tissue processing for ultrastructural analysis. Representative images of callosal fibers from one control (top) and one eAPS mouse (bottom) are shown on the right side (bar corresponds to 1 µm). **b**) The size frequency distributions

for axon and fiber area show a shift to larger axon diameters in eAPS mice. **c**) However, g-ration, axon diameter, fiber diamter and myelin thickness (not shown) did not significantly differ between eAPS and control mice.

Figure 1

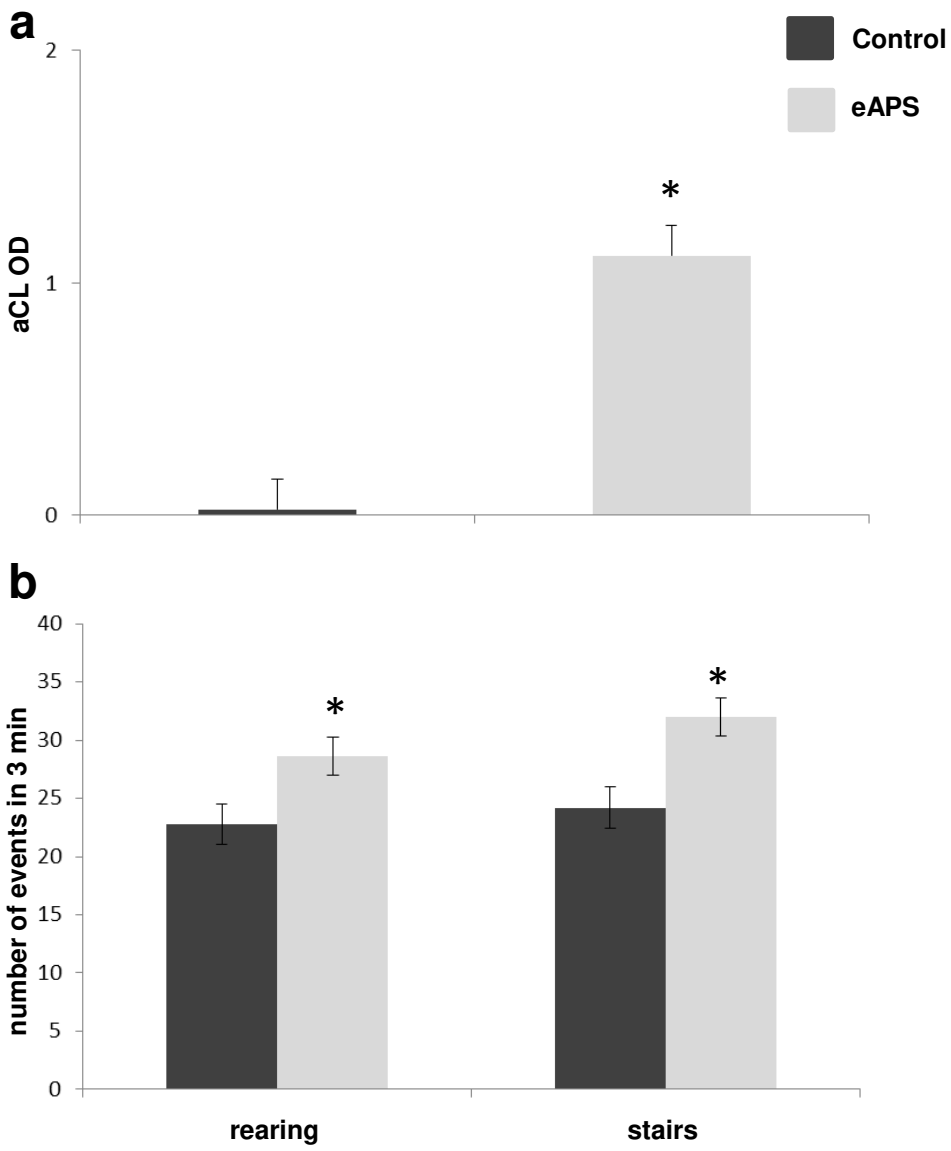
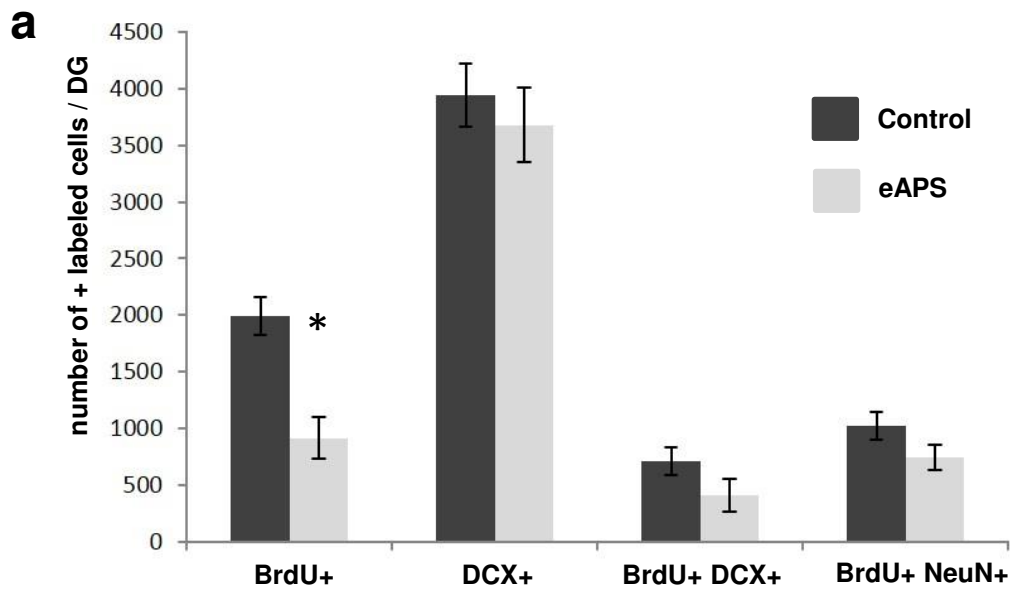
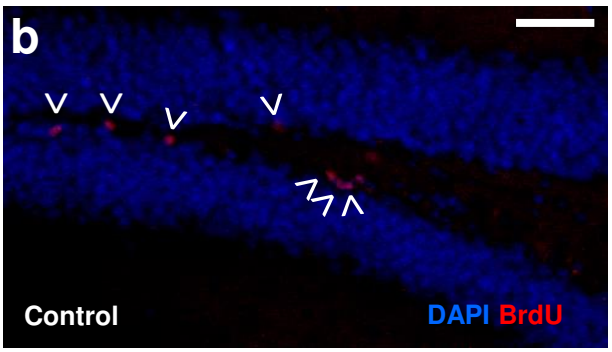
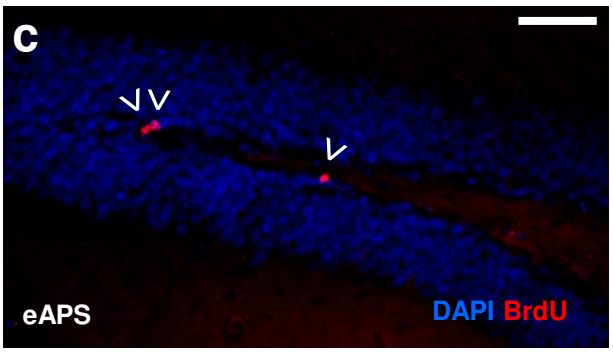

a
Control
eAPS
aCL OD
2
1
0
*
b
number of events in 3 min
40
35
30
25
20
15
10
5
0
*
*
rearing
stairs

Figure 2


a
number of + labeled cells / DG
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
Control
eAPS
BrdU+
DCX+
BrdU+ DCX+
BrdU+ NeuN+


b
Control
DAPI BrdU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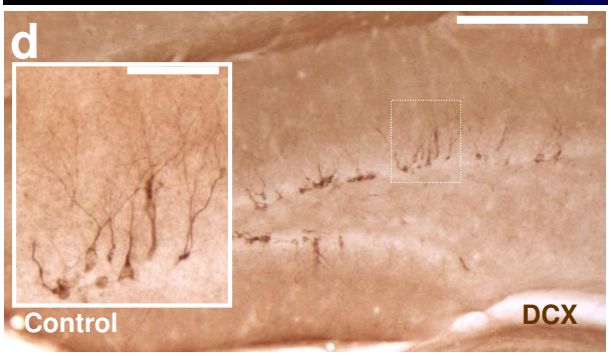
eAPS
DAPI BrdU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Control
DCX

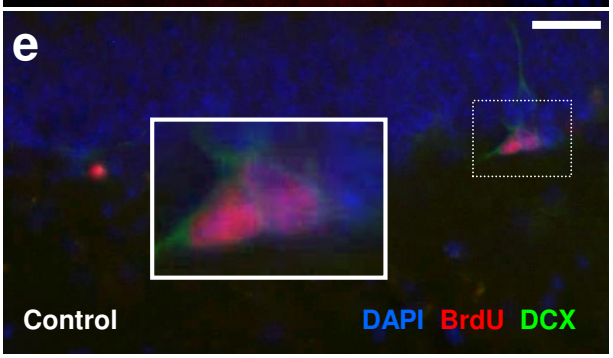
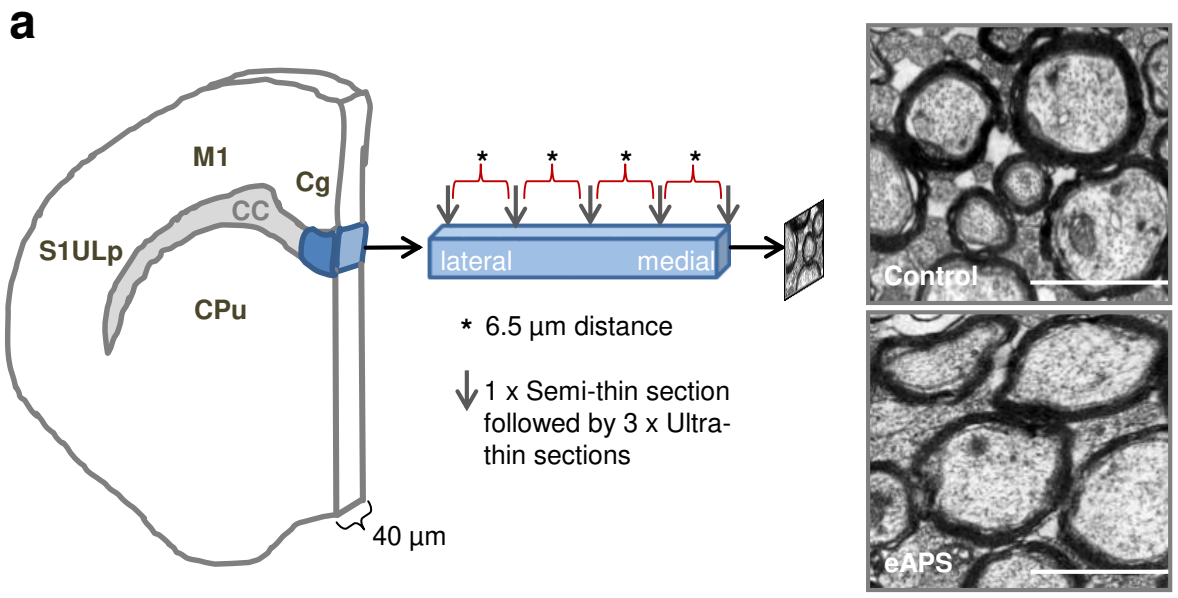
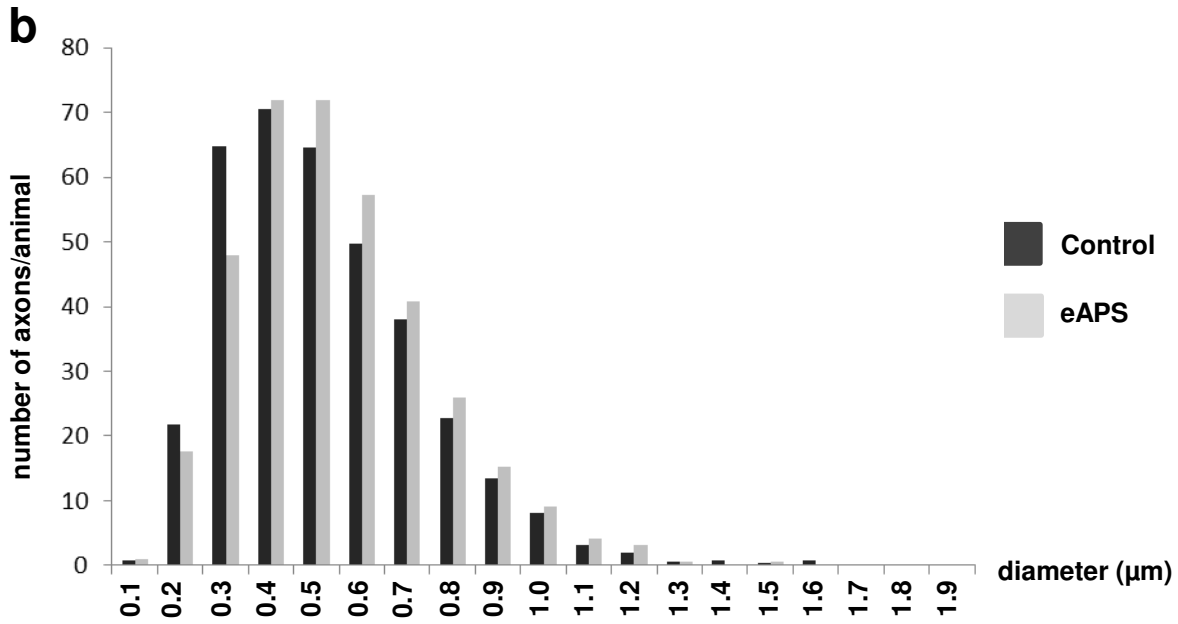

e
Control
DAPI BrdU DCX

Figure 3


a
M1
Cg
CC
S1ULp
CPu
40 µm
lateral
medial
* 6.5 µm distance
1 x Semi-thin section followed by 3 x Ultra-thin sections
Control
eAPS


b
number of axons/animal
80
70
60
50
40
30
20
10
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
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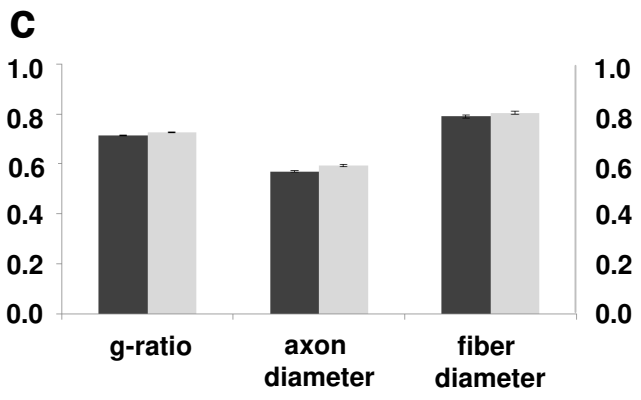
1.3
1.4
1.5
1.6
1.7
1.8
1.9
diameter (µm)
Control
eAPS


c
1.0
0.8
0.6
0.4
0.2
0.0
g-ratio
axon diameter
fiber diameter